Trehalose metabolism in root nodules of the model legume *Lotus japonicus* in response to salt stress

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The effect of NaCl stress (50 mM) and validamycin A treatment (30 μM) on growth and nitrogen fixation of *Lotus japonicus* was investigated in plants cultured under symbiotic and hydroponics conditions for ten weeks (flowering stage). Validamycin A was used as a potent trehalase inhibitor, and was able to produce a five-fold increase in the level of trehalose during salt treatment, concomitant with an enhance in biomass (20%) in salinized plants. Alterations of nodule metabolism related to some carbohydrates and some enzyme activities were also examined. The shoot and total plant dry weight were severely affected by saline conditions decreasing by 40% and only 15–20% in plant treated without or with validamycin A, respectively. Nitrogenase activity (E.C. 1.7.9.92) was inhibited almost 40% by salt stress and no effect of validamycin was observed. Based on these results, *L. japonicus* might be considered as a salt-sensitive legume. In addition, the saline conditions also inhibited the enzyme activities of sucrose synthase (E.C. 2.4.1.13), alkaline invertase (E.C. 3.2.1.26) and trehalose-phosphate synthetase (E.C. 2.4.1.15). The validamycin A treatment mainly decreased enzyme activities: sucrose synthase, trehalose-phosphate phosphatase (E.C. 3.1.3.12) and trehalase (E.C. 3.2.1.28). On the other hand, a high concentration of the carbohydrates, starch, sucrose and glucose, seems not to be the mechanism induced in *L. japonicus* to protect nodules exposed to NaCl because all these sugars decreased in such conditions. Results of the present study support the possible role of trehalose as an osmoprotectant under salt stress.

Introduction

*Lotus japonicus* is a perennial temperate pasture species that is closely related to birdsfoot trefoil (*L. corniculatus* L.) and exhibits characteristics that are useful for genomics, such as a short life cycle, self-fertility, diploidy, relatively small genome size and a short generation time. Phylogenetically, *L. japonicus* is included in the so-called “temperate” or “galegoid” legume group and forms determinate nodules as do “tropical” or “phaseoloid” legumes such as soybean (*Glycine max* L.) and common bean (*Phaseolus vulgaris* L.). It has therefore been proposed to be a model legume for classical and molecular genetic studies on symbiotic plant-microbe interactions (Handberg and Stougaard 1992), and has been demonstrated suitable as a platform for legume crop improvement (Sato and Tabata 2005).

Abbreviations – AI, alkaline invertase; ANA, apparent nitrogenase activity (H₂ production in air, units of μmol H₂ g⁻¹ FW h⁻¹); ANOVA, analysis of variance; DW, dry weight; EDTA, ethylenediaminetetraacetic acid; FW, fresh weight; HK, hexokinase; LSD, least significant difference; NAD⁺, nicotinamide adenine dinucleotide; NDW, nodule dry weight; NFR, nitrogen-fixation rate; PDW, plant dry weight; RDW, root dry weight; SDW, shoot dry weight; SS, sucrose synthase; TPS, trehalose-phosphate synthase; TSS, total soluble sugars; TRE, trehalase.
Soil salinity is one of the major limitations to crop productivity (Epstein 1985) because salinity drastically affects photosynthesis (Sousi et al. 1998), nitrogen metabolism (Cordovilla et al. 1994, Santos et al. 2002), carbon metabolism (Balibrea et al. 2003, Delgado et al. 1993) and provokes disorders in plant nutrition, which may lead to deficiencies of several nutrients (Mengel and Kirkby 2001). Legumes are classified as salt-sensitive crop species (Lauchli 1984) and their production is particularly affected by salt stress because these plants depend on symbiotic N₂ fixation for their nitrogen requirement (Elsheikh and Wood 1995). The limitation in productivity is associated with a lower growth of the host plant, poor symbiotic development of root-nodule bacteria (Georgeiev and Atkins 1993) and consequently with a reduction in the nitrogen-fixation capacity (Delgado et al. 1993). Under stress condition, biochemical and physiological mechanisms, such as accumulation of compatible osmolytes proline, glycine betaine, sucrose and manitol (Zhu 2002), are switched on to protect major processes such as cell respiration, photosynthetic activity, nutrient transport and nitrogen metabolism.

Saline stress has been identified among the main factors affecting growth rate and dry matter production of Lotus species used as forage for animal production in many pastures in temperate regions (Blumenthal and McGraw 1999, Papadopoulos and Kelman 1999). Salt-stressed plants of L. japonicus grown under nitrate nutrition increase up 12-fold proline concentrations in leaves (Márquez et al. 2005), enhance level of oxidative damage (Díaz et al. 2005), but do not change amino acids and protein content and neither glutamine synthetase biosynthetic and Fd-glutamate synthase activities.

Trehalose (α-D-glucopyranosyl-1,1-α-D-glucopyranoside), a non-reducing disaccharide, has been found in a wide variety of organisms such as yeast, fungi, bacteria, plants, insects and other invertebrates. This carbohydrate plays an important role as an abiotic stress protectant stabilizing dehydrated enzymes and membranes as well as protecting biological structures from desiccation damage. In higher vascular plants trehalose may occur in the plants with diseases (Keen and Williams 1969) or colonized by microorganisms, for example in mycorrhizal roots (Harley and Smith 1983), nitrogen-fixing nodules (Streeter 1985) and actinorhizal nodules (Lopez and Torrey 1985). It has been suggested that genetically engineered trehalose accumulation in tobacco plants could improve their drought and salinity tolerance (Romero et al. 1997). Garg et al. (2002) demonstrated that engineering trehalose overproduction can be used to develop new rice (Oryza sativa L.) cultivars with increased abiotic stress tolerance and enhanced rice productivity.

In legumes, Müller et al. (1994) detected large amount of trehalose in root nodules of soybean, being mainly localized (70%) in the bacteroid (Streeter 1987). On the contrary trehalase activity, the only enzyme capable of hydrolyzing trehalose to its glucose monomeric units, has been mainly found in the plant-fraction but relatively low levels in bacteroids (Streeter 1982). In common bean nodules, this enzyme also hydrolyzes sucrose and maltose and other carbohydrates and is severely inhibited (16-fold) by validamycin A treatments (Tejera et al. 2005).

Despite the fact that many groups throughout the world have used this plant for their research, and resources to investigate functional genomics, including mutant collections, are being developed (Colebatch et al. 2004, Márquez 2005), biochemical aspects of the plant in symbiosis deserve more attention. The study of plant and nodule responses under abiotic stresses is of particular interest because Lotus species are important members of plant communities in pastures and their productivity can be seriously affected by saline stress (Díaz et al. 2005, Papadopoulos and Kelman 1999). The objective of the present work was to evaluate the effect of NaCl stress in L. japonicus grown under symbiotic conditions as well as some metabolic and biochemical changes of salt-affected nodules including the possible role of trehalose as an osmoprotectant.

Materials and methods

Plant material and treatments

Lotus japonicus (cv. Gifu) seeds (provided by J. Stougaard and propagated in our laboratory) were scarified by immersion in concentrated H₂SO₄ for 5 min, washed well with sterile water, surface sterilized by immersion in 5% NaClO plus 2% Tween 20 for 20 min and germinated on 0.8% water agar. After 4 days, seedlings were transferred to sterile vermiculite and watered with Hornum nutrient solution (Handberg and Stougaard 1992) with (val+) or without (val−) validamycin A (30 μM) and NaCl (0 and 50 mM). The nutrient solution contained 1 mM KNO₃ only in the first week after sowing and was N-free the remaining time of culture. After 2 days, each seedling (grown in individual pots of about 200 ml) was inoculated with 1 ml of a stationary culture of Mesorhizobium loti R7A strain (ca. 10⁹ cell ml⁻¹) grown in a tryptone-yeast extract medium (Beringer 1974). Plants (60 per treatment) were grown in a controlled-environmental chamber with a 16/8-h light–dark cycle, 23/18°C day night temperature, relative humidity 55/65% and photosynthetic photon flux density (400–700 nm) of 450 μmol m⁻² s⁻¹ supplied by combined fluorescent (Sylvania cool-white lifeline F96T12-CW-VHO, Sylvania
NAD-0.025 U UDP-glucose dehydrogenase and 1.5 M mixtures contained in a volume of 1 ml, 100 M KOH buffer (pH 8.5), 100 M sucrose, 2 M UDP, 0.025 U UDP-glucose dehydrogenase and 1.5 M NaCl and 20% (v/v) ethyleneglycol for sucrose synthase and hexokinase activities. All enzyme activities were expressed per milligram of protein. The assay was stopped by heating at 100°C for 2 min. Samples were centrifuged at 2000 g for 10 min, and the amount of UDP in the supernatant was measured in terms of oxidation of NADH in a linked assay with pyruvate kinase and lactic acid dehydrogenase. The assay mixture contained 50 mM Tris–HCl buffer (pH 8.5), 8 mM UDP-glucose, 30 mM 2-phosphoglycerate, 100 mM MgCl2, 3 mM EDTA and 25 mM KCl. The reaction was started by the addition of the nodule enzyme extract (0.04 ml). After 60 min at 30°C, reactions were stopped by heating at 100°C for 2 min. Samples were centrifuged at 2000 g for 10 min, and the amount of UDP in the supernatant was measured in terms of oxidation of NADH in a linked assay with pyruvate kinase and lactic acid dehydrogenase. The assay mixture contained 50 mM Tris–HCl buffer (pH 7.5), 5 mM phosphoenolpyruvate, 0.24 mM NADH, 10 mM MgCl2, 3.5 U pyruvate kinase and 5 U lactic acid dehydrogenase. The decrease in absorbance at 340 nm was measured continuously over a period of 20 min.

Trehalase-6-phosphate synthetase activity (E.C. 2.4.1.15) was assayed by monitoring the release of UDP from UDP-glucose in the presence of glucose-6-phosphate. The reaction mixture (0.2 ml) contained 100 mM Tris–HCl buffer (pH 7.5), 8 mM UDP-glucose, 30 mM 2-phosphoglycerate, 100 mM MgCl2, 3 mM EDTA and 25 mM KCl. The reaction was started by the addition of the nodule enzyme extract (0.04 ml). After 60 min at 30°C, reactions were stopped by heating at 100°C for 2 min. Samples were centrifuged at 2000 g for 10 min, and the amount of UDP in the supernatant was measured in terms of oxidation of NADH in a linked assay with pyruvate kinase and lactic acid dehydrogenase. The assay mixture contained 50 mM Tris–HCl buffer (pH 7.5), 5 mM phosphoenolpyruvate, 0.24 mM NADH, 10 mM MgCl2, 3.5 U pyruvate kinase and 5 U lactic acid dehydrogenase. The decrease in absorbance at 340 nm was measured continuously over a period of 20 min.

Trehalase-6-phosphate phosphatase activity (E.C. 3.1.3.12) was assayed by monitoring phosphate release from trehalose-6-phosphate (Padilla et al. 2004). The reaction was carried out in a final volume of 0.25 ml containing 25 mM Tris–HCl buffer (pH 7.0), 10 mM MgCl2 and 1 μM trehalose-6-phosphate. Samples were assayed for phosphate by the zinc acetate method (Bencini et al. 1983). All enzyme activities were expressed per milligram of protein.

Organic-solutes analysis

Carbohydrates glucose, sucrose and trehalose were separated and quantified by gas chromatography according to Streeter and Strimbu (1998). For this purpose, samples of nodules (200 mg) were ground in methanol.
(80% v/v) and incubated at 60°C for 10 min followed by centrifugation at 13 000 g for 10 min. The pellet was reextracted three times more, supernatants collected and vacuum dried and resuspended in 0.6 ml of double distilled water. To remove charged compounds, 50 μl of wet mixed-bed ion exchanger (AG1-X8, BioRad, Hercules, CA) was added. The tubes were vortexed and centrifuged (13 000 g, 10 min). The supernatants were transferred to gas chromatography vials and lyophilized. Solids were dissolved in 125 μl of pure pyridine plus 125 μl of Stox reagent (Pierce Biotechnology, Inc., Rockford, IL); this reagent contains hydroxylamine for conversion of anomeric forms to the oxime derivatives and also contains an internal standard, β-phenylglucose. Samples were thoroughly mixed by vortex and were incubated in a 70°C heating block for 30 min with occasional mixing by vortex. After cooling, 200 μl of hexamethyldisilazane and 20 μl of trifluoroacetic acid were added, mixed and allowed to react for 60 min before analysis.

TMS-oxime derivatives were separated on a packed column of 3% OV-17 on Chromosorb WHP using a Hewlett-Packard 5890 Series II gas chromatograph and peak areas were quantified using a Hewlett-Packard 3396A integrator. All reagents were purchased from Pierce Chemical Co. (Rockford, IL).

Starch and total soluble sugars were determined by the colorimetric methods of Kiniry (1993) and Irigoyen et al. (1992), respectively. Samples of nodules (100 mg) were ground in ethanol (70% v/v) and centrifuged at 5000 g for 10 min. For the starch analysis, the insoluble material was dried at 70°C for 24 h, resuspended in 4 ml of distilled water and boiled for 1 h. After cooling, 1 ml of 8.5 mM acetate buffer pH 4.5 was added containing amiloglucosidase (0.8 mg ml⁻¹). To ensure the complete starch hydrolysis, the mixture was incubated overnight at 50°C. Glucose released was determined with the kit ATOM as commented above for alkaline invertase activity assay. The quantification of total soluble sugars was performed on ethanolic extracts incubated with antherase reagent at 100°C for 10 min and the absorbance measured at 625 nm. Both carbohydrates were reported as mg glucose g⁻¹ FW.

Protein concentration in the different extracts was measured at 660 nm by the method of Lowry et al. (1951) using the Folin–Ciocalteau reagent with bovine serum albumin (Sigma-Aldrich, Madrid, Spain) as a standard.

Statistical analyses

The experimental layout was a randomized complete block design. The growth values and parameters related to nitrogen fixation were means of five biological repeats per treatment. Three biological repeats were performed for the enzyme activity assays and the content of protein, total soluble sugar, starch, glucose, sucrose and trehalose. All results were subjected to two-way analysis of variance (ANOVA) with a least significant difference test between means using a Statgraphics 5.0 (Statistical Graphics Corp., Rockville, MD). The standard error and simple correlation coefficients were also calculated.

Results

The effect of NaCl stress on L. japonicus–M. loti symbiosis and the impact of trehalose on nodule metabolism have been examined by altering trehalose levels after addition of validamycin A (a trehalase inhibitor), which prevent trehalose hydrolysis to glucose (Müller et al. 2000).

Growth and nitrogen-fixation parameters

Plant biomass and nitrogen fixation were markedly affected by salt stress conditions (Table 1). NaCl caused a decrease in shoot dry weight (SDW) and total plant dry weight (PDW) about 40% and 15–20% in plants without (0 μM, val–) and with (30 μM, val+) validamycin A, respectively. Under these same treatments, root growth showed a higher salt tolerance than shoot growth only decreasing by 20 and 11%, respectively. All plant growth parameters studied showed a slight decrease (10–18%) with the validamycin A application in the absence of salt. ANOVA results showed that significant differences (95%) of SDW and PDW were because of the salt stress factor.

Regarding ANA the same inhibition was detected (37%) in val+ and val– nodules as result of salt stress but no effect of the validamycin A treatment was observed (Table 1). NFR showed a similar behavior than ANA, a decline of 20% with the presence of NaCl in the growth medium without significant differences in val+ compared with val– nodules. Both ANA and NFR showed a high positive and significant correlation with SDW (r = 0.95* and 0.97**, respectively) and PDW (r = 0.90* and 0.93**, respectively), and significant differences (95%) in both parameters were a consequence of salt stress effect. These correlation values support the close relationship between plant growth and the symbiotic nitrogen fixation of nodules.

Enzyme activities of carbon metabolism

The enzymes sucrose synthase and alkaline invertase, involved in sucrose cleavage, showed a remarkable inhibition by the salinity stress reaching only about 30–55% of the activity in plant untreated with validamycin A (Fig. 1). In addition, the validamycin A treatment produced a decrease in both activities about 10–30% in
Table 1. Shoot dry weight (SDW), root dry weight (RDW), plant dry weight (PDW), nodule dry weight (NDW), apparent nitrogenase activity (ANA) and nitrogen-fixation rate (NFR) in Lotus japonicus inoculated with the Mesorhizobium loti R7A strain and treated with NaCl and validamycin A. Analysis of variance (ANOVA) results are represented as follows: S denotes salinity effect, V denotes validamycin effect and S×V denotes salinity/validamycin interaction effect on a single parameter. Asterisk denotes significant differences at 95%, ns denotes no significant differences. a–d: Means followed by the same letter within a column do not differ (P ≤ 0.05) using the least significant difference (LSD) test. FW, fresh weight.

<table>
<thead>
<tr>
<th>Validamycin A (µM)</th>
<th>NaCl (mM)</th>
<th>SDW (mg plant⁻¹)</th>
<th>RDW (mg plant⁻¹)</th>
<th>PDW (mg plant⁻¹)</th>
<th>NDW (mg plant⁻¹)</th>
<th>ANA (µmol H₂ g⁻¹ FW h⁻¹)</th>
<th>NFR (µmol N₂ g⁻¹ FW h⁻¹)</th>
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<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>283</td>
<td>86</td>
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<td>15.2</td>
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<td>181</td>
<td>69</td>
<td>241</td>
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<td>10.01</td>
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<td>62</td>
<td>284</td>
<td>14.6</td>
<td>2.27</td>
<td>9.85</td>
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<tr>
<td>LSD (0.05)</td>
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<td>11</td>
<td>0.5</td>
<td>0.18</td>
<td>0.80</td>
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</tr>
</tbody>
</table>

ANOVA

S × V

Discussion

The effect of NaCl in the growth of legumes cultured under symbiotic conditions depends greatly on the
sensitivity and/or tolerance to salt of the host plant. Growth parameters SDW, root dry weight (RDW) and PDW of *L. japonicus* were severely affected under saline conditions decreasing until 40% with the NaCl treatment (Table 1). Based on the higher reduction of SDW than RDW under NaCl stress, shoot of *L. japonicus* was more sensitive to salinity than the root as it occurred in grain legumes such as *Cicer arietinum* (Soussi et al. 1998) and *P. vulgaris* (Tejera et al. 2004). However, results also showed a smaller inhibition of SDW and PDW in salinized plants exposed to validamycin A compared with untreated plants (val−), suggesting the possibility that increases in trehalose content of nodules and other organs improve the tolerance of plants in such conditions. In this regard, Müller et al. (1995) and Tejera et al. (2005) did not find any effect of validamycin A on plant growth and nitrogenase activity of soybean and common bean, respectively. However, Müller et al. (1996) reported increases of the trehalose pools size on soybean root nodules subjected to water stress and also found a depression of trehalose accumulation in nodules infected with nitrogen-fixing mutant strains (Müller

<table>
<thead>
<tr>
<th>NaCl Concentration</th>
<th>SS Activity</th>
<th>AI Activity</th>
<th>HK Activity</th>
<th>TPP Activity</th>
<th>TPS Activity</th>
<th>TRE Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM</td>
<td>0.20 µmol NADP⁺·mg⁻¹·prot⁻¹·h⁻¹</td>
<td>2.0 nmol glucose·mg⁻¹·prot⁻¹·h⁻¹</td>
<td>1.2 µmol NADP⁺·mg⁻¹·prot⁻¹·h⁻¹</td>
<td>0.00 nmol Pi·mg⁻¹·prot⁻¹·min⁻¹</td>
<td>0.05 nmol UDP·mg⁻¹·prot⁻¹·h⁻¹</td>
<td>0.00 nmol glucose·mg⁻¹·prot⁻¹·h⁻¹</td>
</tr>
<tr>
<td>50 mM</td>
<td>0.10 µmol NADP⁺·mg⁻¹·prot⁻¹·h⁻¹</td>
<td>1.5 nmol glucose·mg⁻¹·prot⁻¹·h⁻¹</td>
<td>0.9 µmol NADP⁺·mg⁻¹·prot⁻¹·h⁻¹</td>
<td>1.5 nmol Pi·mg⁻¹·prot⁻¹·min⁻¹</td>
<td>0.10 nmol UDP·mg⁻¹·prot⁻¹·h⁻¹</td>
<td>0.5 nmol glucose·mg⁻¹·prot⁻¹·h⁻¹</td>
</tr>
</tbody>
</table>

Fig. 1. Effect of the NaCl treatment on nodule enzymes activities sucrose synthase (SS), alkaline invertase (AI) and hexokinase (HK) of *Lotus japonicus* grown with (val+) or without (val−) validamycin A (30 µM). The data represent the mean values (±standard error, n = 3) of three biological repeats.

Fig. 2. Effect of the NaCl treatment on nodule enzymes activities trehalose-phosphate phosphatase (TPP), trehalose-phosphate synthase (TPS) and trehalase (TRE) of *Lotus japonicus* grown with (val+) or without (val−) validamycin A (30 µM). The data represent the mean values (±standard error, n = 3) of three biological repeats.
Because total plant biomass and nitrogenase activity (ANA, $r = 0.90^*$; NFR, $r = 0.93^*$) were correlated and also showed a similar inhibition, we speculate that negative effect of salt stress on plant growth was a consequence of the decline in the nitrogen-fixation process. Nevertheless, the reduction in growth during salt stress can also be because of osmotic stress, ionic toxicity and nutrient imbalance. Based on our experimental results and the low salt dosage used (50 mM), it could be considered that *L. japonicus* is a salt-sensitive legume.

In our study, most of enzyme activities assayed were more (sucrose synthase, alkaline invertase and trehalose-phosphate synthase) or less (trehalose-phosphate phosphatase and trehalase) affected by the saline conditions (Figs 1, 2). On the contrary, hexokinase behaved as salt-resistant and the validamycin treatment did not noticeably affect the enzyme activity. In soybean sterile roots grown with trehalose added to the grown medium, Müller et al. (1998) found a strong induction of sucrose synthase activity of nodules such as sucrose and maltose and other carbohydrates (Tejera et al. 2005).

A high-concentration starch, sucrose and glucose do not seem to be the mechanism induced in *L. japonicus* to protect nodule functioning in such conditions because these sugars decreased in nodules exposed to NaCl stress (Table 2). In contrast to our result, Müller et al. (1996) detected increased content of sucrose and pinitol in *G. max* root nodules exposed to drought stress; however, in *Vigna unguiculata* and *G. max* nodules the addition of validamycin A caused an increase in the amount of trehalose and a decrease of sucrose and starch (Müller et al. 1995). Curiously, salt and validamycin A increased total soluble carbohydrates of nodules but not those such as starch, sucrose and glucose. The decrease of sucrose concentration presumably was because of the inhibition of sucrose synthase activity because both parameters were highly correlated ($r = 0.95^*$).

Glucose and trehalose together accounted around 1% of the soluble sugar pool detected (Table 2, Fig. 3). Nevertheless, our results indicate an increase (40%) of trehalose content in response to salt stress (Fig. 3) as compared with non-salinized plant, which support the possible role of this disaccharide as an osmoprotectant against abiotic stresses. However, in presence of validamycin A, trehalose content decreased as a consequence of salt treatment (Fig. 3). Based on our experimental results, it is not possible to find an explanation for this opposite behavior, taking into account that biomass and nitrogenase activity (ANA, $r = 0.90^*$; NFR, $r = 0.93^*$) were correlated and also showed a similar inhibition.

### Table 2. Concentration of total soluble sugars (TSS), starch, sucrose and glucose in nodules of *Lotus japonicus* inoculated with the *Mesorhizobium loti* R7A strain and treated with NaCl and validamycin A. Analysis of variance (ANOVA) results are represented as in Table 1. a–d: Means followed by the same letter within a column do not differ ($P \leq 0.05$) using the least significant difference (LSD) test. FW, fresh weight.

<table>
<thead>
<tr>
<th>Validamycin A (μM)</th>
<th>NaCl (mM)</th>
<th>TSS (mg g$^{-1}$ FW)</th>
<th>Starch (mg g$^{-1}$ FW)</th>
<th>Sucrose (mg g$^{-1}$ FW)</th>
<th>Glucose (μg g$^{-1}$ FW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>6.31$^a$</td>
<td>3.19$^d$</td>
<td>2.50$^d$</td>
<td>77.0$^c$</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>9.87$^b$</td>
<td>1.59$^b$</td>
<td>1.20$^e$</td>
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<td>30</td>
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<td>142.2$^d$</td>
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<tr>
<td></td>
<td>50</td>
<td>12.73$^c$</td>
<td>0.77$^a$</td>
<td>1.56$^d$</td>
<td>56.8$^b$</td>
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<tr>
<td>LSD (0.05)</td>
<td></td>
<td>0.46</td>
<td>0.12</td>
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<td>4.8</td>
</tr>
<tr>
<td>ANOVA</td>
<td></td>
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</table>

Fig. 3. Effect of the NaCl treatment on trehalose content in nodules of *Lotus japonicus* grown with (val+) or without (val−) validamycin A (30 μM). The data represent the mean values (±standard error, n = 3) of three biological repeats.
that in both cases (val+ and val−) trehalose-phosphate synthase activity was more inhibited by NaCl when compared with trehalase activity. An enhanced trehalose content of nodules has been also reported in *Medicago sativa* in response to NaCl (more than four-fold, Fougeré et al. 1991), and in *P. vulgaris* (more than two-fold, Farías-Rodríguez et al. 1998) and *G. max* (Müller et al. 1996) under water stress.

The induction of trehalose accumulation in nodules by the validamycin A treatment improved the response of *L. japonicus* under saline conditions increasing significantly PDW (20%) compared with salt-stressed plants cultivated without validamycin application. Nevertheless, the role of this carbohydrate in relationship with the establishment of the symbiosis, connection with other carbon and nitrogen compounds as well as enzyme activities of ammonium metabolism of nodules deserves further experimental analysis. We do not discard other compatible osmolytes such as amino acids and proline (which increase in nodule under salt stress, data not shown), as they could be involved in the protection and/or adaptation to *L. japonicus* to salinity. Previous experiments of Márquez et al. (2005), with nitrate-feed plants, reported up to a 12-fold increase of proline concentration in response to drought and salt stress. Results found in this study allow progress in knowledge of physiological responses of *L. japonicus* to salt stress grown under symbiotic conditions.

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